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Unusual biophysics of immune signaling-related intrinsically disordered proteins

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Intrinsically disordered (ID) regions, the regions that lack a well-defined three-dimensional structure under physiological conditions, are preferentially located in the cytoplasmic segments of plasma membrane proteins, many of which are known to be involved in cell signaling. This is in line with our studies that demonstrated that cytoplasmic domains of signaling subunits of immune receptors, including those of ζ, CD3ε, CD3δ and CD3γ chains of T cell receptor, Igα and Igβ chains of B cell receptor as well as the Fc receptor γ chain represent a novel class of ID proteins (IDPs). The domains all have one or more copies of an immunoreceptor tyrosine-based activation motif, tyrosine residues of which are phosphorylated upon receptor engagement in an early and obligatory event in the signaling cascade. Our studies of these IDPs revealed several unusual biophysical phenomena, including (1) the specific dimerization of disordered protein molecules, (2) the fast and slow dimerization equilibrium, depending on the protein, (3) no disorder-to-order transition and the lack of significant chemical shift and peak intensity changes upon dimerization or interaction with a well-folded partner protein and (4) the dual mode of binding to model membranes (with and without folding), depending on the lipid bilayer stability. Here, I highlight several of these studies that not only facilitate a rethinking process of the fundamental paradigms in protein biophysics but also open new perspectives on the molecular mechanisms involved in receptor signaling.

Introduction

Receptor signaling plays an important role in health and disease. This makes the molecular understanding of signal transduction critical in influencing and controlling this process, thus modulating the cell response. Recently, a novel general platform for receptor-mediated signaling, the Signaling Chain HOmoOLigomerization (SCHOOL) platform, has been suggested and successfully used in drug discovery and early development.[1-3] Within this platform, receptor oligomerization induced or tuned upon ligand binding outside the cell is translated across the membrane into protein oligomerization inside the cell. In the so-called multichain immune recognition receptors (MIRRs), signaling is achieved through receptor-associated subunits that contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains.

Our studies[4-6] revealed that cytoplasmic regions of MIRR signaling subunits, including those of ζ and CD3ε, CD3δ and CD3γ chains of T-cell receptor (TCR), Igα and Igβ chains of B-cell receptor (BCR) as well as the Fc receptor γ chain (ζ_{cyt}, $CD3\varepsilon_{cyt}$, $CD3\delta_{cyt}$, $CD3\gamma_{cyt}$, $Ig\alpha_{cyt}$, $Ig\beta_{cyt}$ and $FcR\gamma_{cyt}$, respectively), represent a novel class of intrinsically disordered proteins (IDPs), the proteins that lack a well-defined three-dimensional structure under physiological conditions. In 2004,[4] we have reported the first evidence of an IDP's propensity for specific homodimerization distinct from protein association in a non-specific manner seen in many systems[7,8] and suggested an important

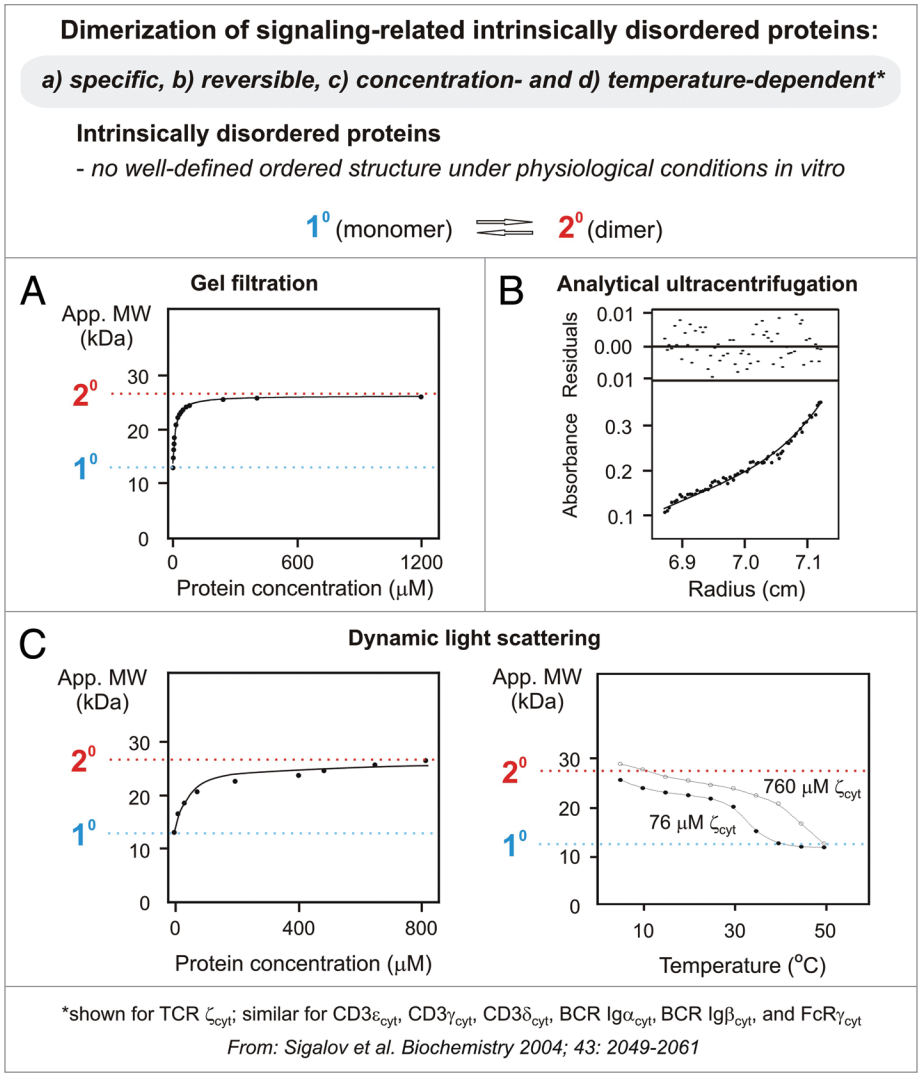

Figure 1. Signaling-related intrinsically disordered proteins can form homodimers in solution and the homodimerization is specific, reversible and concentration- and temperature-dependent as shown by gel filtration (A), sedimentation equilibrium analytical ultracentrifugation (B) and dynamic light scattering. (C) App., apparent; BCR, B-cell receptor; kDa, kilodaltons; MW, molecular weight; TCR, T-cell receptor. Adapted with permission from Sigalov AB, et al. Homooligomerization of the cytoplasmic domain of the T-cell receptor zeta chain and of other proteins containing the immunoreceptor tyrosine-based activation motif. Biochemistry 2004; 43:2049–61.

functional role of IDP homointeractions in transmembrane signaling.[1,3,9,10] Later, in independent studies by several groups, other IDPs have also been found to form specific homodimers[11-14] and shown to function through dimer formation,[11,14] confirming a direct functional link between protein intrinsic disorder and oligomericity.

Thus, our further molecular understanding of receptor signaling critically depends on our understanding of the complex nature and molecular mechanisms of signaling-related IDP interactions. The main purpose of this paper is to attract attention of researchers to our recent studies,[4,5,15-17] that revealed several highly unusual biophysical phenomena, including those that may be unprecedented: (1) the specific dimerization of disordered protein molecules, (2) the fast and slow dimerization equilibrium, depending on the protein, (3) no disorder-to-order transition and the lack of significant chemical shift and peak intensity changes upon dimerization or interaction with a well-folded partner protein and (4) the dual mode of binding to model membranes (with and without folding), depending on the lipid bilayer stability. Intriguingly, these studies not only challenge the generally accepted paradigms in protein biophysics but also open new perspectives on the molecular mechanisms involved in receptor signaling.

Dimerization of Intrinsically Disordered Proteins

The first evidence for the existence of specific homointeractions between IDPs, was reported in 2004[4] when using a variety of biophysical and biochemical techniques, the intrinsically disordered ITAM-containing ζ_{cyt}, CD3ε_{cyt}, CD3δ_{cyt}, CD3γ_{cyt}, Igα_{cyt}, Igβ_{cyt} and FcRγ_{cyt}, all were shown to form specific homodimers (**Fig. 1**).

Table 1. Molecular weight (in kDa) of ζ_{cyt} as calculated[a] and experimentally determined[b] using a variety of analytical techniques

Calculated	ES-MS	Gel filtration	Light scattering		Analytical ultracentrifugation
			static	dynamic	
13.157	13.157	27.1 (1.5)	28.3 (2.7)	25.4 (2.4) 13.2 (1.8)[c]	26.0 (1.3)

Adapted with permission from Sigalov AB, et al. Homooligomerization of the cytoplasmic domain of the T cell receptor zeta chain and of other proteins containing the immunoreceptor tyrosine-based activation motif. Biochemistry 2004; 43:2049–61. [a]The calculated molecular weight was derived from protein sequence. [b]Molecular weight was experimentally determined by electrospray mass spectrometry (ES-MS), gel filtration, static and dynamic light scattering, and sedimentation equilibrium analytical ultracentrifugation. Values in parentheses are the standard errors. Sedimentation equilibrium data assume that standard errors for each data point are equal to the square root of the χ^2-values. [c]In the presence of 5 M guanidinium chloride (GdnHCl).

Gel filtration chromatography is widely used for determination of the size and molecular weight (MW) of proteins under native conditions, and it is a useful technique to monitor molecular association/dissociation. Our gel filtration experiments performed using a broad range of protein loading concentrations revealed that the IDPs studied are predominantly dimeric in solution suggesting the specificity of homooligomerization (**Fig. 1A**; shown for ζ_{cyt}, similar for CD3ε_{cyt}, CD3δ_{cyt}, CD3γ_{cyt}, Igα_{cyt}, Igβ_{cyt} and FcRγ_{cyt}).[4] To avoid the pitfalls associated with using only a single technique to study a given phenomenon, other methods, such as sedimentation equilibrium analytical ultracentrifugation (AUC) and dynamic light scattering (DLS), were also used. AUC allows direct determination of absolute MW, independent of shape. Using this technique, it is possible to determine both the stoichiometry and equilibrium constants for the species present. DLS is a rapid and nondestructive method to measure the hydrodynamic properties of proteins as they freely diffuse in solution. Using gel filtration, AUC and DLS, we determined the apparent MW of ζ_{cyt} (**Fig. 1 and Table 1**). As compared to the MW predicted from the sequence and confirmed by mass spectroscopy (13,157 Da), the native apparent MW indicated the formation of oligomeric forms, with the predominant species being a dimer.

The temperature dependence DLS experiments (**Fig. 1C**) revealed that for ζ_{cyt} at 76 μM, the apparent MW changed from about 25 kDa at lower temperatures to approximately 12 kDa at higher temperatures, with the transition occurring between 30 and 40°C.[4] The temperature-induced transition range for ζ_{cyt} is concentration-dependent, tending to higher temperature values with increasing protein concentration. At 760 μM, the transition range starts at about 40°C, approximately 10°C higher than for ζ_{cyt} at 76 μM. The protein remains soluble under all conditions studied, and the temperature-induced dimer dissociation is reversible for both ζ_{cyt} concentrations, as shown by decreasing temperature from 50 to 5°C, which results in reversal of the temperature-induced changes.[4]

Taken together, the data obtained suggest that a random coil ζ_{cyt} is predominantly dimeric and the oligomerization is reversible and dependent on concentration and temperature (**Fig. 1 and Table 1**).[4]

Similar results were observed for cytoplasmic domains of the other ITAM-containing proteins, namely CD3ε_{cyt}, CD3δ_{cyt}, CD3γ_{cyt}, FcRγ_{cyt}, Igα_{cyt} and Igβ_{cyt}.[4] As compared to the MW values predicted from the sequences and confirmed by mass spectroscopy, the native apparent MWs determined using gel filtration and DLS suggest the formation of predominantly dimeric species for each of these proteins in a wide range of protein concentrations (**Table 2**). Addition of guanidinium chloride (GdnHCl) resulted in dissociation of oligomers, with apparent MWs values determined by DLS in the presence of 5 M GdnHCl close to those measured by mass spectroscopy and corresponding to monomers (**Table 2**). The observed GdnHCl-induced oligomer dissociation was reversible and removal of GdnHCl by dialysis resulted in increasing of apparent MW values to those corresponding to dimers. We have shown also that completely phosphorylated ζ_{cyt} and FcRγ_{cyt} (six and two moles of phosphate per mole of protein, respectively) are dimeric in solution.[4]

Thus, on the example of signaling-related IDPs, our study[4] demonstrated, for the first time, that disordered protein molecules can homodimerize and this dimerization is specific, reversible and concentration- and temperature-dependent. Later, the natural propensity of ζ_{cyt} to homodimerize was independently confirmed by other investigators.[18] Finally, in further independent studies by several groups,[11-14] homodimerization was confirmed for other IDPs extending the phenomenon to different classes of IDPs and suggesting its physiological relevance. It should be noted that in most of these studies, dimerization is accompanied by a mutual or "synergistic"[19] folding of two IDP molecules at the interaction interface.[10] Thus, interactions between the constituents of such homodimers represent specific interactions between folded regions involved in complex formation.

Perspectives. The cytoplasmic homointeractions of signaling-related IDPs have been suggested as a novel therapeutic target for a variety of disorders.[9,20,21] In this context, the recent success in modulation of FcγRIIA signaling by using agents that target cytoplasmic homointeractions[22] clearly demonstrates the technological feasibility of the suggested strategy[9,20,21] to modulate receptor signaling as well as its fundamental and clinical importance. Also important in this respect is the recent recognition of IDPs, in general, as potential drug targets.[23]

However, despite the unusual biophysical phenomenon of IDP homooligomerization was first reported in 2004,[4] and since that has become of more and more interest

Table 2. Molecular weights (in kDa) of cytoplasmic domains of the other ITAM-containing proteins studied as calculated[a] and experimentally determined[b] using a variety of analytical techniques

Protein	Calculated	ES-MS	Gel filtration	Dynamic light scattering	
				w/o GdnHCl[c]	5 M GdnHCl
CD3ε_{cyt}	6.323	6.325	12.5 (1.4)	11.9 (2.2)	7.6 (1.8)
CD3δ_{cyt}	5.140	5.140	8.6 (1.2)	9.4 (2.1)	5.6 (0.8)
CD3γ_{cyt}	5.370	5.372	11.8 (1.4)	10.8 (2.4)	6.1 (1.0)
FcRγ_{cyt}	5.010	5.010	11.1 (1.4)	9.6 (1.4)	4.6 (0.5)
phospho-FcRγ_{cyt}	5.186	5.182	12.1 (1.6)	10.5 (1.6)	5.3 (0.6)
Igα_{cyt}[d]	7.142	7.142	18.3 (2.0) 37.4 (3.1)	19.7 (2.8)	8.5 (1.9)
Igβ_{cyt}	5.654	5.654	12.3 (1.4)	10.2 (2.3)	5.0 (0.7)

Adapted with permission from Sigalov AB, et al. Homooligomerization of the cytoplasmic domain of the T cell receptor zeta chain and of other proteins containing the immunoreceptor tyrosine-based activation motif. Biochemistry 2004; 43:2049–61. [a]The calculated molecular weights were derived from protein sequences. [b]Molecular weights were experimentally determined by electrospray mass spectrometry (ES-MS), gel-filtration and dynamic light scattering. Values in parentheses are the standard errors. [c]Guanidinium chloride. [d]The values determined for dimeric and tetrameric Igα_{cyt} are indicated.

to biophysicists and biochemists,[24,25] at the current state of our knowledge, the molecular mechanisms of IDP homooligomerization are not well understood. As a result, dimerization/oligomerization interface(s) are still not characterized at the residual level, thus impeding design of specific agents that target these protein-protein interactions. One can expect that further multidisciplinary studies will shed light on the possible structural basis of these interesting IDP features and significantly assist in the development of novel and effective therapies.

Fast and Slow Dimerization Equilibrium

In gel filtration experiments, the observed gradual shift of retention volume with increasing protein concentration, rather than an appearance of new peaks and a change in the intensity of peaks corresponding to monomer and dimer, indicates a fast dynamic equilibrium between monomeric and dimeric ζ_{cyt} species (**Fig. 2A**).[4] For CD3ε_{cyt}, CD3δ_{cyt}, CD3γ_{cyt}, FcRγ_{cyt} and Igβ_{cyt} (but not Igα_{cyt}), the gel filtration patterns and the concentration dependence of retention times and therefore of apparent MW values are similar to those observed for ζ_{cyt} (**Figs. 1A and 2A**).[4] This indicates that there exists a fast dynamic equilibrium between monomeric and dimeric cytoplasmic domains of all of the ITAM-containing IDPs studied (but not Igα_{cyt}, see below).

Further, cross-linking should theoretically trap the oligomers that are normally in a dynamic equilibrium with the monomers. In contrast to the gel filtration profile of non-cross-linked ζ_{cyt} at the same loading protein concentration, the profile of the BS^3 cross-linked ζ_{cyt} sample (**Fig. 2A**, the inset) exhibits separated peaks, with retention volumes corresponding to those of the monomeric and dimeric forms (**Fig. 2A**).[4] Most of the cross-linked ζ_{cyt} remains as a monomer, suggesting that the degree of cross-linking obtained in this experiment disrupts the non-covalent self-association of this protein, possibly due to the steric constraints resulting from intramolecular cross-link formation and/or chemical modification of the residues involved in protein-protein interactions. The difference in gel filtration patterns for non-cross-linked and cross-linked ζ_{cyt} further supports the existence of a fast dynamic equilibrium between monomeric and dimeric ζ_{cyt} species in solution.

In contrast to the other ITAM-containing proteins studied, two well-separated peaks were observed by gel filtration for Igα_{cyt} with apparent MWs corresponding to monomeric and dimeric species at low protein concentration, and to dimeric and tetrameric species at higher protein concentration (**Fig. 2B**).[4] Even below 10 μM, where the other ITAM-containing proteins are mostly monomeric, Igα_{cyt} is mostly dimeric. The amount of tetrameric form increased with increasing protein loading concentration, and this equilibrium is slowly reversible.[4] No differences in retention time values were observed for the isolated and re-injected peaks corresponding to tetrameric and dimeric Igα_{cyt} (**Fig. 2B**). These results suggest that for Igα_{cyt} the dynamic equilibrium between monomeric and oligomeric species is significantly slower and the protein-protein interaction is stronger than those observed for other proteins studied.

Perspectives. The individual contribution of Igα and Igβ to BCR-triggered fates is unclear. It is known that in the mIg-Igα-Igβ BCR complex, Igα tends to bind more readily to Src family members than does Igβ,[26] and it activates tyrosine kinases more efficiently.[27,28] It remains unclear if this feature of Igα may be attributed to the unique ability of its cytoplasmic domain to form stable homooligomers in solution (mostly, dimers and tetramers) even at very low protein concentrations where Igβ_{cyt} is mostly monomeric.[4] Further studies will be required to test whether the observed difference in the oligomerization behavior of Igα and Igβ can provide a solid biophysical foundation on which to build our improved understanding the functioning of the Igα-Igβ heterodimer and possibly to develop novel therapeutic approaches targeting BCR signaling.

No Disorder-to-Order Transition upon Dimerization

The generally accepted view in protein biophysics is that upon binding to their

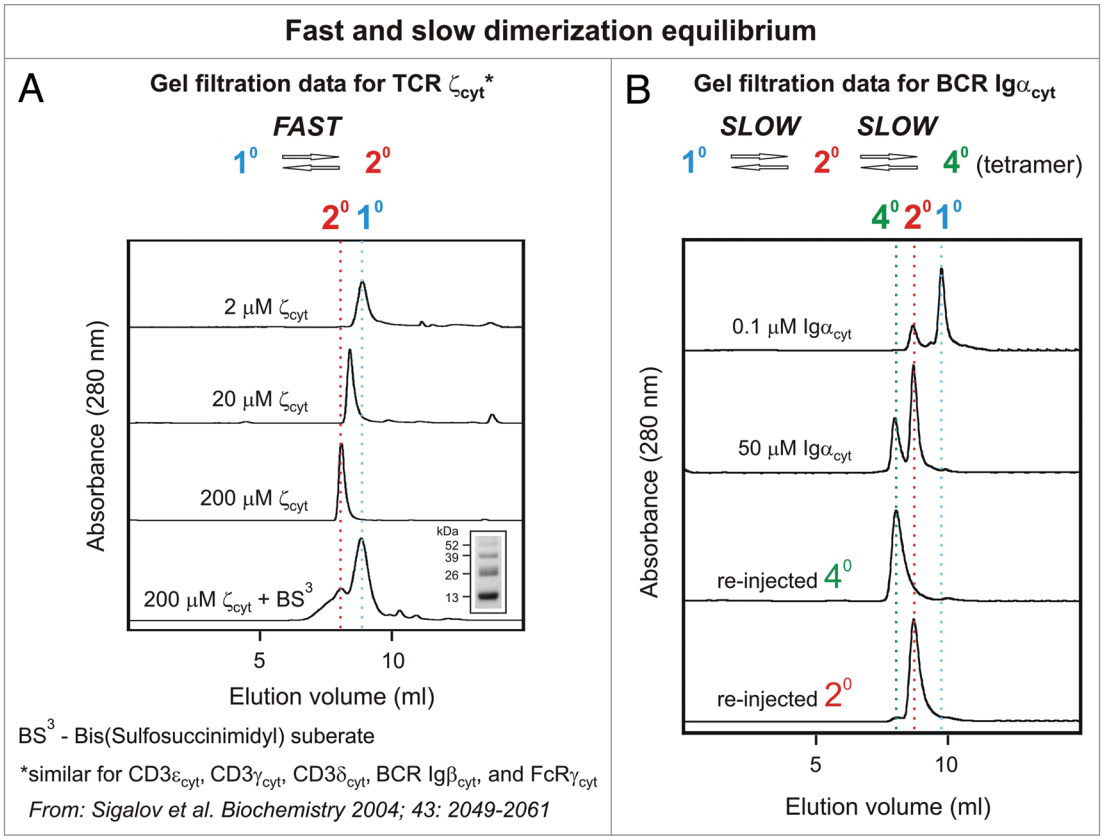

Figure 2. Depending on the protein, homooligomerization of signaling-related intrinsically disordered proteins is characterized by fast (A) or slow (B) dimerization equilibrium as demonstrated by gel filtration. The oligomers that are normally in a dynamic equilibrium with the monomers can be trapped by chemical cross-linking as shown by gel filtration analysis of the BS^3 cross-linked ζ_{cyt} sample (A and the inset). BCR, B cell receptor; TCR, T cell receptor. Adapted with permission from Sigalov AB, et al. Homooligomerization of the cytoplasmic domain of the T cell receptor zeta chain and of other proteins containing the immunoreceptor tyrosine-based activation motif. Biochemistry 2004; 43:2049-61.

interacting partners and targets, IDPs undergo transitions to more ordered states or fold into stable secondary or tertiary structures—that is, they undergo a disorder-to-order structural transition upon binding.[6,10,29-32]

Experimentally, protein disorder can be detected by far-UV circular dichroism (CD) spectroscopy that allows the estimation of the secondary structure content of a protein in solution. Our CD analysis of CD3ε_{cyt}, CD3δ_{cyt}, CD3γ_{cyt}, FcRγ_{cyt}, Igα_{cyt} and Igβ_{cyt} shows the characteristics of IDPs in both monomeric and dimeric forms (**Fig. 3**).[4,5] For ζ_{cyt} and FcRγ_{cyt}, no changes were observed in the CD spectra when the proteins were completely phosphorylated (**Fig. 3**).[4,5] Considering that the CD spectra for both protein forms are very similar, it can be concluded that oligomerization does not induce any secondary or tertiary structure formation. Thus, intermolecular interactions in these dimers represent specific interactions between disordered protein molecules.

Perspectives. IDPs are often referred to as "remaining predominantly disordered" or "largely unfolded" upon dimerization or interaction with other proteins or lipids,[11-14,32-39] meaning that the protein regions flanking the interaction interface but not the interface itself remain disordered. Recently, it has been suggested to term this mode of interaction "the flanking fuzziness" in contrast to "the random fuzziness" when the IDP remains entirely disordered in the bound state.[40] In this context, "the flanking fuzziness" is a part of the "coupled binding and folding" paradigm.

Our studies of a novel class of signaling-related IDPs demonstrate, for the first time, that binding of IDPs is not necessarily accompanied by a disorder-to-order structural transition even within the interaction interface,[4,5,15-17] thus going beyond the classical paradigm. The ITAM-containing IDPs are directly involved in receptor-mediated signaling, which makes these findings particularly interesting and important. Future studies should aim at defining the nature and precise molecular mechanisms of the "no disorder-to-order transition" mode of interactions of IDPs.

No Chemical Shift and Peak Intensity Changes upon Binding

In recent years, nuclear magnetic resonance (NMR) spectroscopy has become uniquely useful in the experimental characterization of IDPs and is currently the most powerful approach to probing the conformational dynamics and interactions of IDPs.[31,41-49] In CD spectroscopy, while for an ordered protein the CD signal gives information about each molecule in the sample, because nearly all the molecules are in the same structural state, it

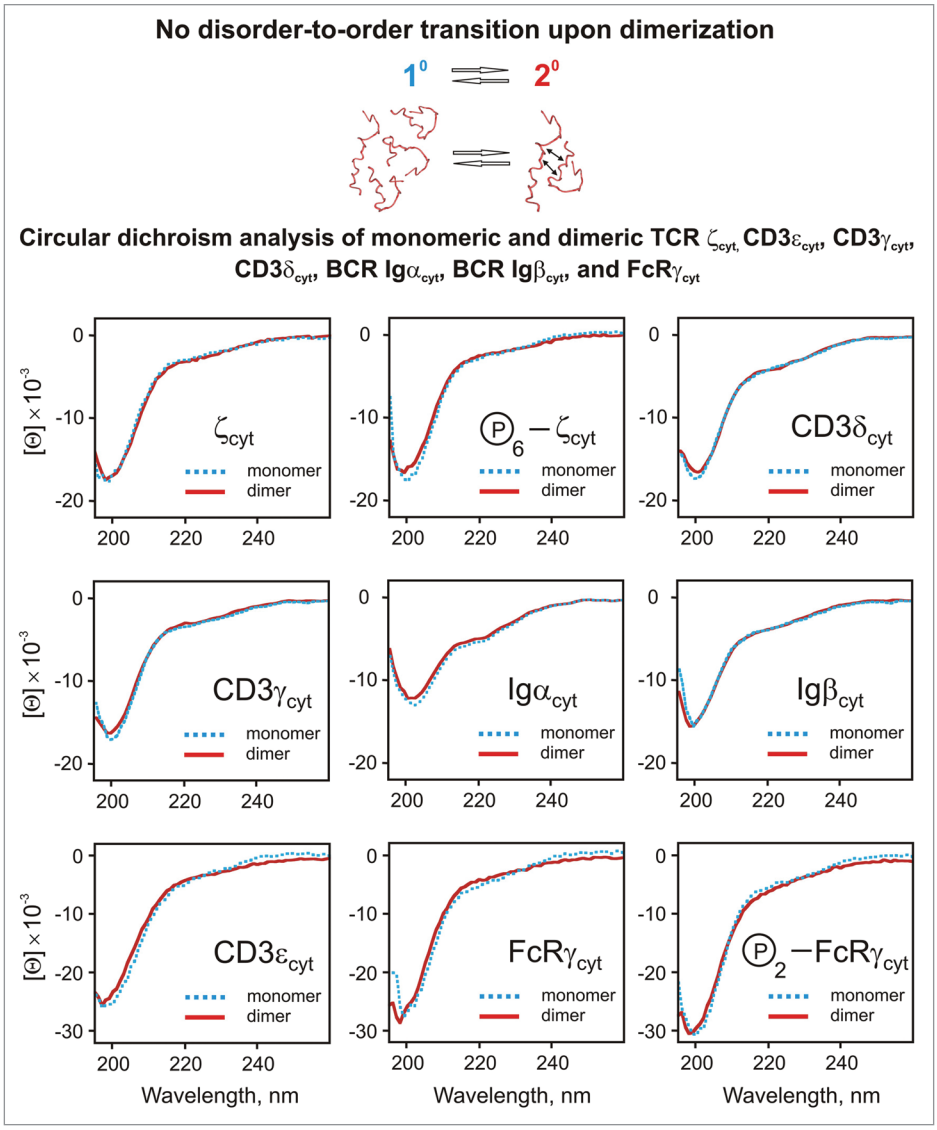

Figure 3. Dimerization of signaling-related intrinsically disordered proteins is not accompanied by a disorder-to-order structural transition as shown by circular dichroism. No folding is also observed for fully phosphorylated ζ_{cyt} and FcRγ_{cyt} (six and two moles of phosphate per mole of protein, respectively). BCR, B-cell receptor; TCR, T-cell receptor. Adapted with permission from Sigalov AB, et al. Lipid-binding activity of intrinsically unstructured cytoplasmic domains of multichain immune recognition receptor signaling subunits. Biochemistry 2006; 45:15731–9.

is different for an IDP that consists of a broad ensemble of molecules each having a different conformation.[49] In this context, NMR is unparalleled in its ability to provide detailed structural and dynamic information on IDPs and has emerged as a particularly important tool for studies of IDP folding and interactions.[31,41-49]

NMR chemical shifts and line widths are extremely sensitive to subtle changes in protein conformational ensembles and are indispensable for detecting protein disorder (poor proton chemical shift dispersion is indicative of disorder), determining propensities of secondary structure formation on a residue-by-residue basis in unfolded and partly folded proteins and mapping protein-protein interactions.[50] For the latter purpose, it is important that just as the ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectrum represents a fingerprint of the amide backbone of a protein, the ^{1}H-^{13}C HSQC spectrum is considered as a fingerprint of the aromatic side chains.[51]

Dimerization. In our studies,[4,16,17,52] we expressed and purified ^{15}N-labeled and ^{15}N,^{13}C-double labeled ζ_{cyt} for our NMR experiments aiming to characterize the conformational dynamics of this 13-kDa IDP and map the interaction interface(-s) upon ζ_{cyt} dimerization or binding of a random-coil ζ_{cyt} to a folded partner protein. Using our low temperature strategy, we were able to overcome common challenges faced in assigning HSQC spectra of IDPs and fully assign the ^{1}H,^{15}N-HSQC spectrum of free ζ_{cyt}.[4,52] The low signal dispersion of the ζ_{cyt} backbone amide ^{1}H chemical shifts is typical for IDPs (**Fig. 4**, blue peaks).[4]

First, we used heteronuclear NMR spectroscopy and the ^{15}N,^{13}C-double labeled recombinant ζ_{cyt} protein[17] to monitor the conformational state of ζ_{cyt} on a

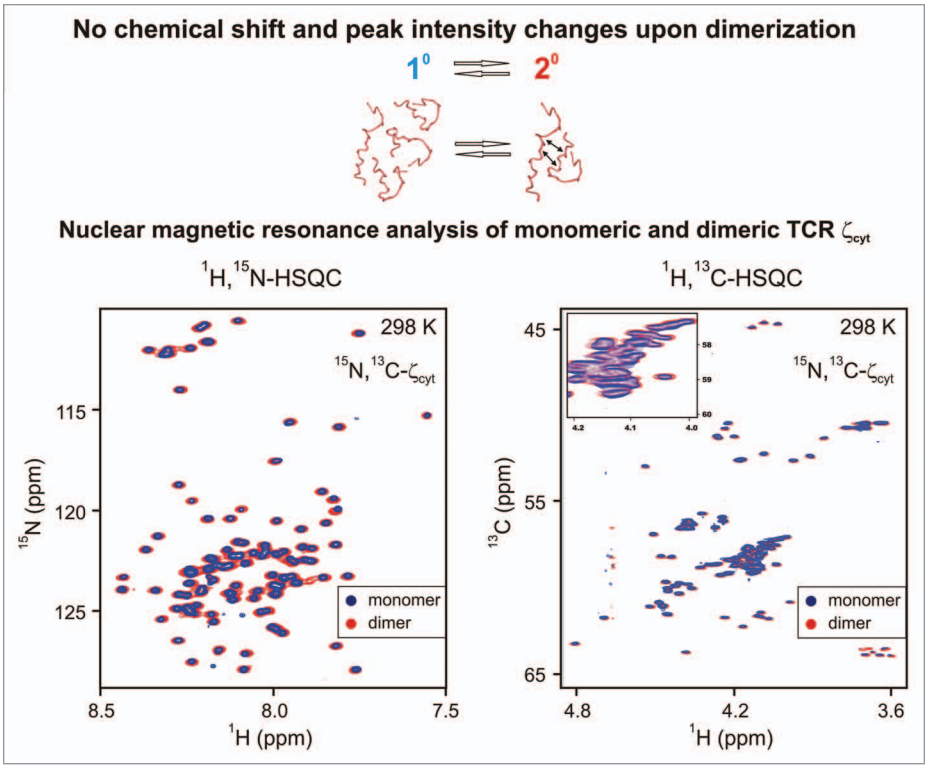

Figure 4. No significant chemical shift and peak intensity changes are observed in the $^{1}H,^{15}N$- and $^{1}H,^{13}C$-HSQC spectra of the $^{15}N,^{13}C$-double labeled random coil ζ_{cyt} protein upon dimerization. Abbreviations: HSQC, heteronuclear single quantum correlation; TCR, T cell receptor. Adapted with permission from Sigalov AB, et al. Binding of intrinsically disordered proteins is not necessarily accompanied by a structural transition to a folded form. Biochimie 2007; 89:419–21.

per residue level and further confirm our previous CD and NMR data (**Fig. 3**)[4,5] indicating that dimerization of ζ_{cyt} is not accompanied by a disorder-order structural transition. Another aim of our NMR experiments was to characterize the dimerization interface(s) of ζ_{cyt}.[17]

Intriguingly, NMR studies of ζ_{cyt} dimer[4,17] revealed a new, previously unrecognized NMR phenomenon—the lack of significant changes in chemical shift and peak intensity upon a specific protein complex formation.[4,17] No chemical shift changes and significant changes in peak intensities are observed in the ^{1}H-^{15}N and the ^{1}H-^{13}C HSQC spectra of $^{15}N,^{13}C$-double labeled ζ_{cyt} upon dimerization (**Fig. 4**).[17] Careful analysis of the few minor differences between the spectra revealed that those differences can be explained by differences in sensitivity or minor changes in sample conditions. Importantly, the protein fraction close to 100% is visible at both high and low ζ_{cyt} concentrations, where the protein is predominantly in the dimeric and monomeric forms, respectively.[17]

Together with our previous CD and NMR studies,[4,5] these findings further confirm that a random coil ζ_{cyt} does not undergo a disorder-to-order structural transition upon dimerization and thus remains unfolded both outside and within the dimerization interface(s).[17] On the other hand, it seems hard to believe but our NMR study[17] indicates, for the first time, that the formation of protein complex is not necessarily accompanied by changes in chemical shifts and peak intensities in the fingerprint HSQC spectra, thus challenging the use of these spectra for mapping the interaction interface(s).

Binding to a well-folded partner protein. The simian immunodeficiency virus (SIV) Nef-ζ_{cyt} complex represents a novel system to study the interaction of an IDP with a well-structured partner. We applied gel filtration and heteronuclear NMR spectroscopy to study binding of ζ_{cyt} to the well-folded SIV Nef core domain and to characterize the conformational state of Nef-bound ζ_{cyt} on a per-residue level.[16] Considering surprising results of our NMR study of the ζ_{cyt} homodimer,[17] we have developed a general experimental strategy that allows us to address possible questions and concerns related to the validity of the experimental data and analysis (**Fig. 5**).[16]

In order to study the Nef-ζ_{cyt} interaction and its binding stoichiometry, we used a gel filtration assay developed and optimized as previously described for the ζ_{cyt} dimerization studies.[4] The gel filtration analysis was performed for Nef and uniformly ^{15}N-labeled ζ_{cyt} alone as well as for the Nef-ζ_{cyt} mixtures at constant ζ_{cyt} concentration of 15 μM and 1:1, 1:2 and 2:1 molar ratios of Nef to ζ_{cyt} (**Fig. 6A**).[16] The apparent native MW for ζ_{cyt}, determined as 26.2 kDa using a range of MW markers for calibration, is consistent with that previously reported for a ζ_{cyt} dimer.[4] The apparent MW of 17.7 kDa determined for the SIV Nef core domain corresponds to that predicted by the protein sequence (16,679 Da), while the apparent MW of 31.6 kDa determined for the Nef-ζ_{cyt} complex is consistent with that predicted for

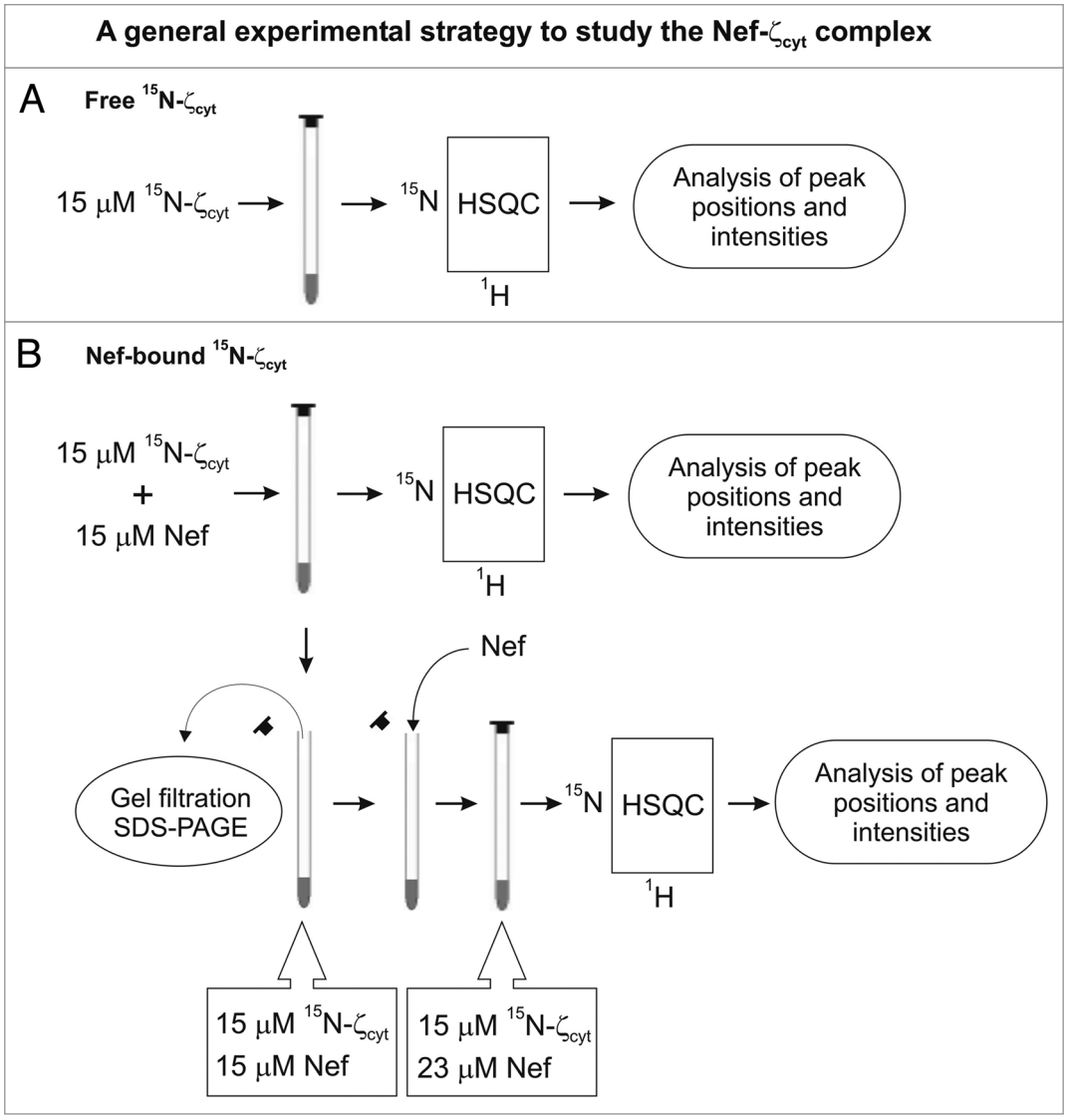

Figure 5. A general experimental strategy used to study the Nef-ζ_{cyt} complex. HSQC, heteronuclear single quantum correlation; Nef, negative factor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate. Adapted with permission from Sigalov AB, et al. The intrinsically disordered cytoplasmic domain of the T cell receptor zeta chain binds to the nef protein of simian immunodeficiency virus without a disorder-to-order transition. Biochemistry 2008; 47:12942–4.

an equimolar complex (**Fig. 6A**). SDS-PAGE profile of the Nef-ζ_{cyt} complex (**Fig. 6A**, the inset) also confirms a 1:1 molar ratio. The Nef-ζ_{cyt} binding affinity apparently is higher than that of ζ_{cyt} dimerization, previously reported to be about 10 μM,[4] and considering the approximate twenty-fold sample dilution through the chromatographic column, is likely to be less than 1 μM.[16]

In our NMR experiments,[16] all but three of the expected cross-peaks were observed in the HSQC spectrum of free ζ_{cyt} (**Fig. 6B**, blue peaks) that has been fully assigned previously.[4,52] No substantial changes in the average peak intensity were found in the free and Nef-bound ζ_{cyt} samples indicating that essentially all of the ^{15}N-labeled ζ_{cyt} in the Nef-ζ_{cyt} sample is observed under these NMR conditions. Surprisingly, the overlaid spectra of free and Nef-bound ζ_{cyt} show no differences in chemical shift values for all observed ζ_{cyt} residues (**Fig. 6B**).[16] Similar results were obtained for ζ_{cyt} in the presence of 1.5-fold excess of Nef.[16] Importantly, while the ζ_{cyt} dimerization interface(s) is not yet known, two SIV Nef interaction domains (SNIDs) on ζ_{cyt} that comprise portions of the first and second of the three ITAMs (**Fig. 6B**) are well-established,[53] but residues in the SNIDs also do not exhibit chemical shift and peak intensity changes upon binding (**Fig. 6B**; cross-peak positions of SNID residues are marked).[16]

Thus, the lack of Nef-induced changes in cross-peak chemical shifts and intensities provides strong evidence that ζ_{cyt} remains unfolded upon binding to Nef.[16] These findings are in line with our previous NMR studies of a ζ_{cyt} homodimer where no chemical shift changes were observed upon dimerization,[4,17] indicating no disorder-to-order structural transition during this process. Importantly, this work[16] provides

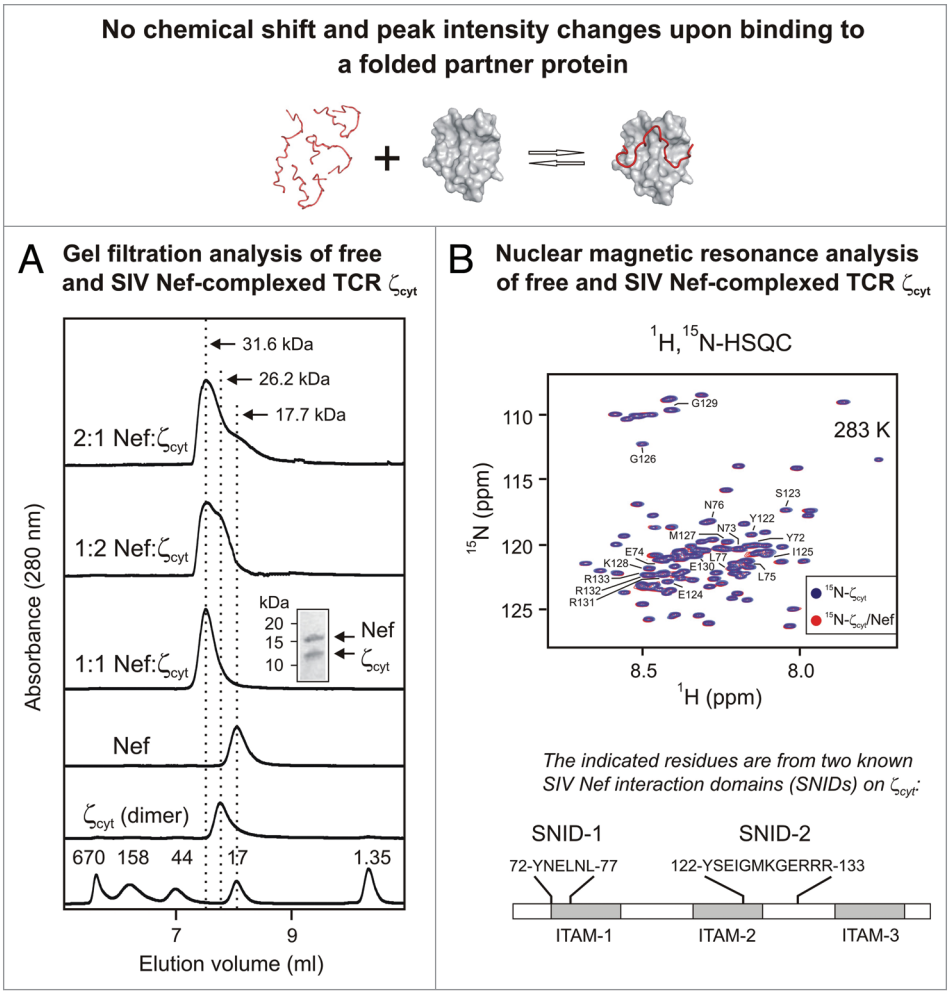

Figure 6. Intrinsically disordered ζ_{cyt} forms a 1:1 complex with the well-folded SIV Nef protein as shown by gel filtration and SDS-PAGE (A and the inset). Upon binding, no significant chemical shift and peak intensity changes are observed in the $^{1}H,^{15}N$-HSQC spectra of the SIV Nef-complexed ^{15}N-labeled ζ_{cyt} protein, including those residues that are known to be important for binding (B). HSQC, heteronuclear single quantum correlation; Nef, negative factor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SIV, simian immunodeficiency virus; SNID, SIV Nef interaction domain; TCR, T cell receptor. Adapted with permission from Sigalov AB, et al. The intrinsically disordered cytoplasmic domain of the T cell receptor zeta chain binds to the nef protein of simian immunodeficiency virus without a disorder-to-order transition. Biochemistry 2008; 47:12942–4.

further strong evidence that the formation of protein complex is not necessarily accompanied by changes in chemical shifts and peak intensities in the fingerprint HSQC spectra, thus eliminating possible artifactual explanations of the unprecedented NMR phenomenon observed.[4,16,17]

Perspectives. ^{1}H-^{15}N HSQC spectra represent a fingerprint of the protein backbone and are widely used as a quick, informative probe of changes in backbone conformation, particularly as it relates to structural studies of IDPs and their complexes.[43,45] Thus, the unique and unprecedented NMR phenomenon observed, the lack of significant changes in chemical shifts and peak intensities upon binding of IDP to another IDP or to a folded partner protein,[4,16,17] likely highlights an unusual nature of specific interactions of IDPs upon binding without folding on their targets and opens a new line of research in protein biophysics. These interactions can play important roles in cellular signaling and viral pathogenesis.[9,54] Thus, defining the nature of these unique interactions and developing ways to control and influence them can have not only fundamental but also practical impact.

Dual Mode of Binding to Lipid Bilayers

Considering a crucial role of ζ_{cyt}, CD3ε_{cyt} and FcRγ_{cyt} in immune signaling and their close proximity to the cell membrane, the question whether or not membrane binding of these IDPs can promote folding of their ITAMs and thus lead to inaccessibility of the ITAM tyrosines for phosphorylation is of fundamental importance in our understanding of receptor triggering. However, little is known about lipid-binding activity of the ITAM-containing cytoplasmic domains and the existing data are strikingly contradictory,[5,6,32] suggesting that ζ_{cyt}, CD3ε_{cyt} and FcRγ_{cyt} ITAMs fold[6,32] or do not fold[5] upon binding to lipid bilayers.

Our recent study[15] resolves this contradiction and reveals, for the first time, a dual mode of binding of IDPs to lipid bilayers (**Fig. 7**): (1) binding followed by helical folding of the ITAMs

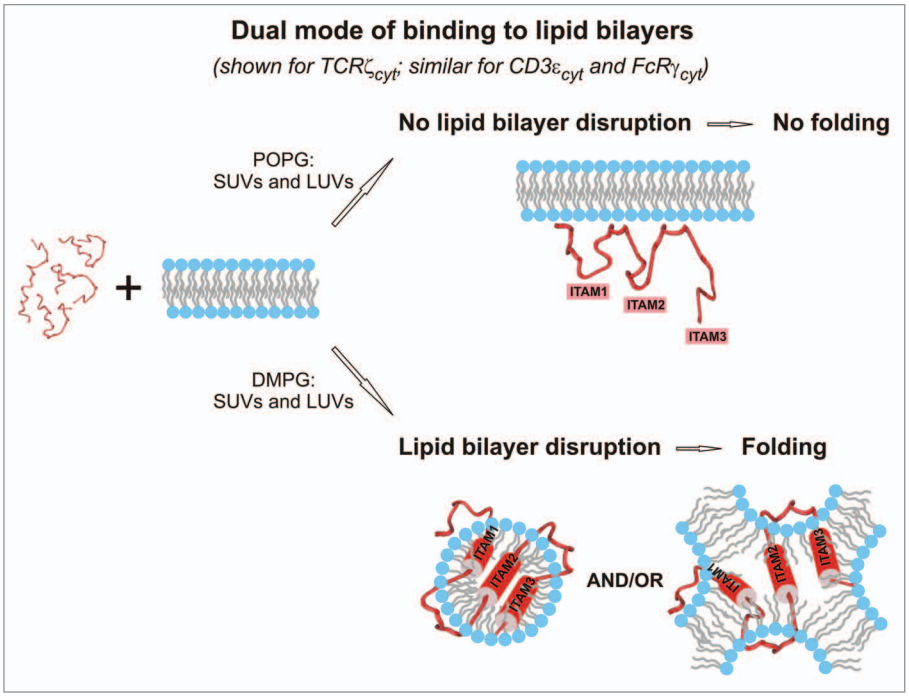

Figure 7. Depending on the lipid bilayer stability, binding of signaling-related intrinsically disordered proteins may or may not be accompanied by a disorder-to-order structural transition. Abbreviations: DMPG, dimyristoylphosphatidylglycerol; LUVs, large unilamellar vesicles; SUVs, small unilamellar vesicles; POPG, 1-palmytoyl-2-oleoyl-sn-glycero-phosphatidylglycerol; TCR, T-cell receptor. Adapted with permission from Sigalov AB, Hendricks GM. Membrane binding mode of intrinsically disordered cytoplasmic domains of T cell receptor signaling subunits depends on lipid composition. Biochem Biophys Res Commun 2009; 389:388–93 and Sigalov AB. The SCHOOL of nature. II. Protein order, disorder and oligomericity in transmembrane signaling. Self/Nonself 2010; 1:89–102.

(this was shown for small and large unilamellar vesicles of dimyristoylphosphatidylglycerol, DMPG SUVs and LUVs, respectively) and (2) binding without a disorder-to-order structural transition (this was shown for SUVs and LUVs of palmytoyl-2-oleoyl-sn-glycero-phosphatidylglycerol, POPG, which would be expected to be a better model to mimic the cell membrane than DMPG vesicles). The study demonstrates that binding of highly positively charged ζ_{cyt}, $CD3\varepsilon_{cyt}$ and $FcR\gamma_{cyt}$ (net charges are +5, +11 and +3, respectively) to acidic phospholipids destabilizes and disrupts DMPG lipid bilayers.[15] Using electron microscopy and DLS, we have shown that upon protein binding, both SUVs and LUVs of DMPG fuse and rupture.[15] In contrast, stable POPG vesicles remain intact under these conditions.[15] Thus, when using DMPG vesicles as a model membrane, protein binding-induced membrane perturbation and disruption can represent a molecular basis for the observed formation of the ITAM helixes[6,15,32] questioning the relevant models suggested for receptor triggering.[6,32,55]

Perspectives. Importantly, our findings[15] demonstrate that depending on lipid type, the use of vesicles of the same size and surface charge can result in opposite conclusions regarding membrane-binding activity of proteins and its physiological relevance. This provides, for the first time, molecular explanation of the current discrepancies in the literature and highlights the importance of ensuring the integrity of model membranes upon protein binding.[15,56] Most importantly, this study[15] demonstrates how the use of an inappropriate membrane model can result in misleading conclusions regarding membrane-binding activity of proteins in the cell membrane. Thus, efforts should be made in both the choice of an appropriate membrane model and in the experimental design of lipid-protein interaction studies, especially in studies of IDPs.

Conclusions

In conclusion, intrinsically disordered cytoplasmic domains of signaling subunits of immune receptors, namely ζ_{cyt}, $CD3\varepsilon_{cyt}$, $CD3\delta_{cyt}$, $CD3\gamma_{cyt}$, $Ig\alpha_{cyt}$, $Ig\beta_{cyt}$ and $FcR\gamma_{cyt}$, represent a novel class of IDPs. As such, these ITAM-containing proteins can be used in a variety of biophysical and biochemical studies as a convenient system of functionally relevant IDPs. Our studies of these IDPs have already revealed several highly unusual biophysical phenomena that facilitate a rethinking process of the fundamental paradigms in protein biophysics and open a new line of research in this field.

Further, within the SCHOOL platform of immune signaling,[1-3] formation of competent signaling homooligomers in cytoplasmic milieu is necessary and sufficient to trigger receptor activation. In single-chain receptors (i.e., those receptors in which binding and signaling domains are located on the same protein chain), cytoplasmic regions represent folded and well-ordered domains. In the context of SCHOOL signaling, this suggests the principal functional link between protein order and oligomericity in cytoplasmic milieu.[10,57] In contrast, in MIRRs (i.e., those receptors in which binding and signaling domains are located on separate subunits), the ITAM-containing cytoplasmic domains of signaling subunits all are intrinsically disordered, suggesting the principal functional link between protein disorder and oligomericity.[10,57]

In my opinion, we encounter here one of the most intriguing questions: Why for MIRRs, the receptors that transduce the extracellular ligand-binding information into specific intracellular signaling events through associated signaling chains, did nature select to use a functional link between protein disorder and oligomericity? One can expect that further multidisciplinary studies of signaling-related IDPs will not only explain unusual biophysical properties of these proteins but also lay the foundation for clarifying this question of great interest and practical utility.

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